Reference: *Biol. Bull.* **178:** 25–32. (February, 1990)

Correlation of Abnormal Radular Secretion with Tissue Degrowth During Stress Periods in *Helisoma trivolvis* (Pulmonata, Basommatophora)

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Abstract. Laboratory experiments on starvation stress in *Helisoma trivolvis* elucidate a relationship between modifications of radular secretion and tissue degrowth resulting from stress. Tissue losses in starved adults ranged from 4.5% at 40 days to 27.7% at 160 days, with negligible mortality (<2%). Modifications in radular secretion that paralleled tissue loss involved not only abnormal secretion of individual teeth and of tooth rows, but especially an increased "packing" of radular rows per unit ribbon length. Radular length remained constant during experimental trials, however the mean number of tooth rows increased by almost 47% after 120 days of food deprivation. Radular patterns reflecting degrowth observed in these experiments were paralleled in radulae taken from overwintered animals sampled from natural populations. Rates of radular turnover averaged between 2.3% new growth per day (43 days to turnover) and 4.0% new growth per day (25 days to turnover). Radular samples could provide for *post hoc* detection of recent periods of tissue degrowth in snails, just as evidence of longer periods of tissue degrowth can be detected in the shells of long-lived bivalves.

Introduction

Natural populations of aquatic molluscs can experience tissue loss during winter. This phenomenon involves complex shifts in metabolism and has been called "degrowth" (Russell-Hunter, 1985). Previous reports have demonstrated that physiological stress, both short-term and of longer duration, can temporarily affect radular secretion (Isarankura and Runham, 1968; Kerth, 1971; Fujioka, 1985; Smith, 1987). The laboratory experiments reported here were designed to clarify the relationship between abnormal radular secretion and concurrent tissue degrowth resulting from starvation stress. Applied to field populations, these observations could provide an independent (short-term) method for detecting periods of starvation that had occurred shortly before sampling. This would complement the long-term detection of stress-induced degrowth based on "oversized" shells (Russell-Hunter *et al.,* 1984), or on modified catabolism (Russell-Hunter *et al.,* 1983). The Ramshorn snail of eastern North America, *Helisoma trivolvis* (Say, 1817), was particularly suitable for these experiments because it performs well in laboratory culture, and because recent studies have documented not only the biometry and mechanics of its radula (Smith, 1987, 1988, 1989), but also its actuarial bioenergetics, including its capacity for degrowth (Russell-Hunter and Eversole, 1976; Russell-Hunter *et al.,* 1983, 1984).

Detailed analysis of radula-tooth biometry in *Helisoma* has shown significant levels of interpopulation variation in this species (Smith, 1987, 1989). As with similar studies on Lymnaeid pulmonates by Berrie (1959) and Hunter (1975), in *Helisoma* there are no observable ecophenotypic effects on tooth shape. Despite this constancy (within individuals, and within populations) more general aspects of radular secretion, including the number and density of tooth rows, can be modified by environmental stress. Short exposures to near-freezing temperatures will produce a zone of modified tooth rows on the radula ribbon (Isarankura and Runham, 1968; Kerth, 1971; Fujioka, 1985; Smith, 1987), and longer exposures to the stress of starvation will produce "bunching" or "packing" of radular rows, as described below.

Received 6 July 1989; accepted 14 November 1989.

Work on the bioenergetics of tissue degrowth in *Helisoma* is also cognate to these experiments. Held in the laboratory in a metabolic framework simulating that of natural overwintering, a representative cohort of *Helisoma* showed a 50% loss of tissue biomass (involving perhaps 20% loss of protein) with only 10% mortality over 132 days (Russell-Hunter and Eversole, 1976). Metabolic shifts during the degrowth process were studied by Russell-Hunter *et al.* (1983) using nearly concurrent assessments of oxygen consumption and of nitrogenous excretion. There was a clearly controlled differential catabolism of protein resources during degrowth. One of the first quantitative reports of direct field evidence for tissue degrowth during winter is for natural populations of *Helisoma trivolvis* and of *Lymnaea palustris* in central New York state (Russell-Hunter *et al.*, 1984).

The existence of all these recent reports not only made stocks of *Helisoma* appropriate for these experiments, but also made it likely that a history of recent degrowth could be detected by detailed examination of the radula. In field studies this might come to parallel evidence (Clark, 1976; Mallet *et al.*, 1987; Peterson *et al.*, 1985) of longer periods of degrowth and regrowth which can be detected in the shells of long-lived bivalves.

Materials and Methods

Helisoma trivolvis is one of the more common gastropod molluses of central New York state. This euryoecic, pulmonate snail is found primarily in eutrophic environments including lakes, ponds, streams, farm ponds, and drainage ditches. Mature adults used for laboratory analysis of tissue degrowth were taken from a small pond in Ithaca, New York (76°22.96′W, 42°25.78′N). To study the effects of overwintering on radula secretion, as well as the patterns of radular regrowth during early spring, snails were sampled in April and May, 1985, from four additional field sites (Eaton Reservoir, 75°42.27′W, 42°51.10′N; Meadowbrook Pond, 76°07.08′W, 43°01.59′N; Otter Pond, 76°32.83′W, 43°09.52′N; and Silver Lake, Remsen, 75°08.19′W, 43°20.97′N).

Methods used to quantify tissue degrowth were modified from those of Russell-Hunter and Eversole (1976), and protocols for radula preparation are detailed by Smith (1987). To initiate an investigation of tissue degrowth, more than 400 adult snails were collected at the Ithaca field site in October 1985. This sample reflects the natural variation in a single generation of *Helisoma* as it moves into winter conditions. All shells were measured with dial calipers (±0.1 mm) for maximum shell diameter (MD). On the basis of MD, individuals were then divided into three size classes: <14.0, 14.1–16.9, and >17.0 mm. Two individuals from each class were then cultured together in translucent plastic beverage cups (n = 6 per container) in approximately 400 ml of filtered pond water. At this time, groups, each containing six snails, were randomly designated (using Japanese icosahedral dice) as "fed" or "starved" experimentals or as baseline controls. Fed animals were provided fresh lettuce for the duration of the experiment; starved animals were starved for 120 days and then fed lettuce for the last 40 days of the trial. The experiment was run in a B.O.D. chamber at 8°C. Cold fluorescent lights illuminated the cultures on a 14L/10D cycle. Cups were cleaned, and provided with fresh, filtered, water each week.

Samples were taken at 0, 40, 80, 120, and 160 days both for analysis of tissue degrowth and for radular preparations. Of 264 animals used in this study, 72 were designated controls and 192 were experimentals. Of these 192, 144 were used for tissue analysis and 48 were used for estimating radular degrowth. At 40-day intervals, samples of 18 snails from each treatment were assessed for shell and tissue dry weight. Individuals were oven dried at 65°C, treated with an excess of 8.5% HNO_3 (12% v/v nitric acid), washed, and then redried, giving two dry weights, whole snail and tissue, and, by subtraction, a value for dissolved calcium carbonate. Tissue degrowth was then calculated as the difference between actual tissue dry weight (TDW) and that predicted from initial tissue-to-shell regressions established at the start of the trial (TDWp).

To determine the effects of food deprivation on radular secretion, 6 specimens from each of the above treatments were sampled every 40 days. Individuals were sacrificed in boiling water and removed from their shells. The buccal mass of each individual was removed, softened in saturated KOH for 2–5 seconds, and transferred to distilled water. Radulae were then removed with fine forceps, placed onto clean glass slides, arranged, and covered with coverglasses. Preparations were then held in distilled water for 24 hours, dehydrated in 70% EtOH and air dried. New coverglasses and mounting fluid were then applied. Abnormal radular secretion was quantified by first dividing each radula into three equal sectors on the basis of overall length. The total number of tooth rows per sector was then counted.

To study the natural patterns of return to normal radular secretion following overwinter stress, adult *Helisoma* were collected in early spring at four field sites. Sampling continued until evidence of abnormal secretion (row-packing) was no longer present. Radular growth rates were calculated as length of new growth as a fraction of ribbon length.

Methods used to study radular turnover in the laboratory follow Isarankura and Runham (1968). Pond water was cooled to approximately 1°C. Snails were placed in this bath for 24 hours. Individuals were then returned to room temperature (18°C) and were provided fresh lettuce. Individuals were sacrificed daily until regions of radular malformation were absent.

Table I

Tissue degrowth in Helisoma trivolvis

A.	40 d	80 d	120 d	160 d
Fed	−1.9 ± 1.63	−6.5 ± 1.41	−8.7 ± 1.50	−11.3 ± 1.91
Unfed	−2.2 ± 1.54	−8.6 ± 1.62	−13.1 ± 1.69	−14.6 ± 1.80
B.	40 d	80 d	120 d	160 d
Fed*	−3.5 ± 2.58	−11.0 ± 2.46	−15.7 ± 2.29	−19.0 ± 3.05
Unfed**	−4.5 ± 2.57	−15.6 ± 3.44	−23.8 ± 2.05	−27.7 ± 2.83

* ANOVA $F_{3,68} = 7.438$, $P < 0.001$

** ANOVA $F_{3,68} = 13.878$, $P < 0.001$

Data were subject to arcsin-square root transformation before analysis.

A. Change in tissue dry weight in milligrams (as TDW-TDWp, n = 18) over 160 days. B. Change in tissue dry weight as a percentage of predicted tissue dry weight [((TDW-TDWp)/TDWp)·100, n = 18], over 160 days.

Results

At the start of the laboratory trial 72 control individuals had been sacrificed. For these, analysis showed that tissue dry weight (mg) related to shell dry weight (mg) as TDW = 0.254·SDW + 1.335 (r = 0.956, n = 72, $P < 0.001$). With each set of known values of SDW, this relationship was then used as a predictor of TDW. The deviation of predicted (TDWp) from expected TDW for each individual was used as an indicator of tissue growth or of tissue degrowth. These values for each of four sampling periods are set out in Table I. Two-hundred and sixty-four individuals began the trial; four died (<2%) and were replaced with parallel experimental animals. Tissue degrowth clearly occurred by 80 days in both sets of experimental animals, and this had nearly doubled by 160 days (Table I). Degrowth over 160 days of food deprivation ranged from 4.5% tissue loss at 40 days to 27.7% tissue loss at 160 days. These values correspond to 2.2 mg below predicted tissue dry weight and 14.6 mg below TDWp, respectively. Degrowth in animals belonging to the fed treatment ranged from 3.5% at 40 days to 19.0% at 160 days. Levels of tissue loss in fed and unfed treatments did not differ ($P > 0.05$) at 40 and at 80 days. At 120 and at 160 days, however, treatments did show significantly different mean levels of degrowth (t_{120} days = 2.414, n = 36, $P < 0.05$; t_{160} days = 2.098, n = 36, $P < 0.05$).

Tissue degrowth in the experimental snails was paralleled by abnormal radular secretion (Fig. 1). This was manifest not only in malformations of individual radular rows (including smaller lateral, marginal, and rachidian teeth; irregular lateral, marginal, and rachidian teeth; and missing marginal teeth), but also, and most consistently, by an increased number of radular rows (packing) per unit ribbon length. Radular length remained constant during experimental trials, however the mean number of tooth rows increased by almost 47% after 120 days of food deprivation (Table IIa). Observations showed this increase was associated with the generative (posterior) end of the radular ribbon. Although secretory activity of the odontoblasts continued during the trial, secretion by the membranoblasts (which produces lengthening of the radula ribbon) proceeded at a much reduced relative rate (Fig. 2). [The precise mechanism of post-secretory radular transport remains uncertain. The topic has been reviewed by Runham (1963), Mischor and Märkel (1984), and Mackenstedt and Märkel (1987).] This differential activity of odontoblasts and of membranoblasts resulted in an increased density of tooth rows at the posterior end of the radula ribbon (Fig. 2). During the last 40 days of the trial (refeeding), radular transport was restored and the proper pattern of radular secretion was again established (Table IIb). Once a normal pattern of secretion was established, the region between the tightly compressed rows (generated during stress) and the normally deposited rows (generated during refeeding) provided a marker which could be used to quantify radular turnover rates either in experimental or in natural populations.

Patterns of abnormal radular secretion observed in the laboratory were paralleled in radulae taken from animals sampled from five field sites (Fig. 3). Weekly sampling in early spring allowed a unique opportunity to estimate radular growth and turnover rates under natural conditions. Values among five sites ranged between 3–4% new growth per day. This figure corresponds to approximately 5–7 rows per day, and to 225–315 teeth per day. Radulae from Meadowbrook Pond showed the slowest turnover (2.3% growth/day, 43 days to turnover) while radulae from Ithaca turned over most rapidly (4.0%/day, 25 days to turnover). Radulae from the other three sites turned over in approximately 30 days (Eaton, 2.9%/day, 34 days turnover; Otter, 3.6%/day, 28 days turnover, Remsen, 3.5%/day, 29 days turnover). These rate data agree with laboratory trials, which showed ribbon turnover in 30 days at room temperature. Field rates also agree with those determined by Isarankura and Runham (1968) who reported average radular production of approximately 3.2 rows per day (minimum 0.5, maximum 7.5, average minimum 2.8, average maximum 4.2 rows/day) for a variety of molluscs including *Helix* and *Lymnaea*. At one field site (Ithaca) the progress of return to normal radular secretion correlated with changes in tissue regrowth (as tissue/shell) (r = 0.937, n = 5, $P < 0.05$) indicating that radular growth is associated with increases in early spring tissue biomass.

Discussion

There is no doubt that, for *Helisoma trivolvis* at least, our experimental conditions closely match those of over-

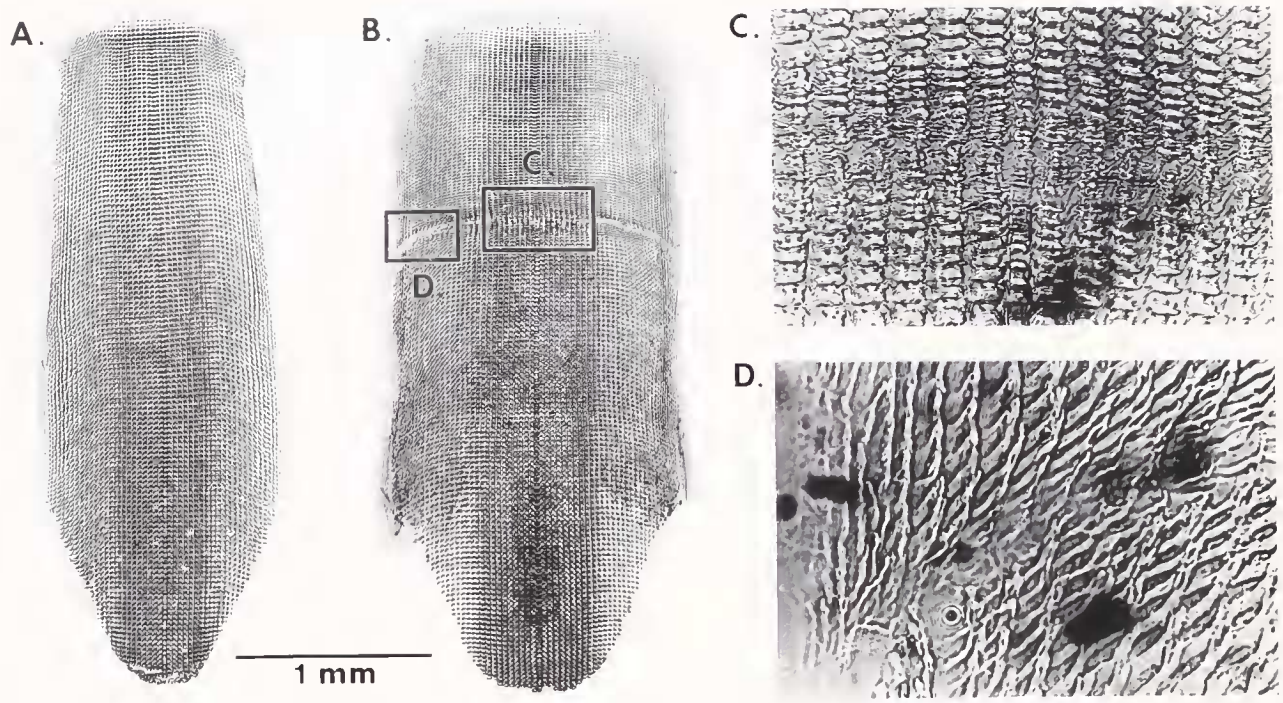

Figure 1. Untouched photographs to show radular malformations in *Helisoma*. A. Normal radula. B. Radula after 75 days of food deprivation. In both A and B, the most recently secreted radular rows are at the top of each photograph, and the rows in use are near the bottom. C. Enlarged view of rachidian and lateral-tooth region from B. Note lateral tooth malformations and row packing. D. Enlarged view of marginal-tooth region from B. Note that marginal teeth are absent from the region of degrowth.

wintering in natural populations. Degrowth in three field populations measured by Russell-Hunter *et al.* (1984) showed average losses in tissue biomass of 24.7%, 28.3%, and 41.3%. The maximum loss of 27.7% over 160 days in these experiments is appropriate.

The results of the present investigation confirm that the physiological stress of starvation in *Helisoma* results not only in tissue degrowth but also in concurrent changes in radular secretion. The significance of this concurrence is twofold, involving first, possible insight into the fundamental control mechanisms of stress response in molluscs, and second, the possibility (for applied studies) of *post hoc* detection, in natural populations, of earlier periods of starvation or similar stress. Before reviewing these two aspects, however, it is necessary to set out certain strengths and weaknesses in laboratory starvation experiments.

In general, quantitative studies of any kind of stress on

Table II

Modification of radular secretion in Helisoma trivolvis

A.	0 d	40 d	80 d	120 d	160 d
Length (mm)*	2.7 ± 0.04	2.9 ± 0.16	2.7 ± 0.17	2.7 ± 0.19	2.4 ± 0.29
Total rows**	138 ± 1.9	169 ± 9.4	192 ± 9.8	202 ± 13.7	194 ± 27.1
Rows/mm***	52 ± 0.9	59 ± 1.3	70 ± 2.0	77 ± 4.1	82 ± 9.7

* ANOVA $F_{4,20} = 0.707$, $P > 0.5$, **ANOVA $F_{4,20} = 3.072$, $P < 0.05$, ***ANOVA $F_{4,20} = 6.724$, $P < 0.01$.

B.	0 d	40 d	80 d	120 d	160 d
Anterior	34.6 ± 0.40	30.6 ± 0.51	28.8 ± 0.92	26.0 ± 1.48	31.4 ± 3.23
Middle	32.2 ± 0.37	29.2 ± 0.97	27.6 ± 0.40	24.4 ± 1.17	32.8 ± 4.89
Posterior	33.2 ± 0.49	40.6 ± 0.87	43.4 ± 1.03	49.2 ± 2.25	36.2 ± 2.82
Row number	138 ± 1.9	169 ± 9.4	192 ± 9.8	202 ± 13.7	194 ± 27.1

A. Basic statistics for abnormal radular secretion in unfed snails over 160 days (n = 5). B. Sector analysis as percent per sector based on total number of rows (average total on last line).

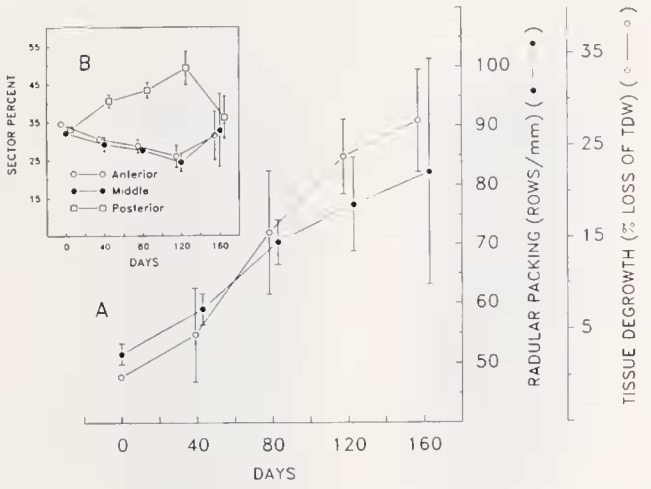

Figure 2. Patterns of radular modification and tissue degrowth in laboratory stocks of *Helisoma trivolvis*. Main plot (A) shows correlation of tissue degrowth and abnormal radular secretion. Insert plot (B) shows pattern of radular packing (see text for further explanation). Vertical bars are 95% confidence limits of each mean.

animals in laboratory culture must not involve high rates of mortality. Our survivorship rate (>98%) in the experimental groups is clearly satisfactory. Evidence of degrowth in snails and in other shelled molluscs is based on the permanence of the calcareous shell as a record of previous tissue biomass. In a review of molluscan degrowth studies, Russell-Hunter (1985) emphasizes an important *caveat*, that ratio measurements of tissue-shell relationships and, hence, predicted values of tissue biomass should be obtained only from those species that demonstrably show no shell resorption. *Helisoma trivolvis* has been well studied in this respect, and its shell does not change in mass or in composition (Russell-Hunter and Eversole, 1976; Russell-Hunter *et al.*, 1983, 1984).

A more immediate difficulty is that, with experimental groups set up as in the present series, it is empirically impossible to provide polar trophic conditions. Under our experimental conditions, "fed" snails are not satiated, while "unfed" snails are not totally starved (microorganisms are present in 7-day-old water and on shells). Our controls represent unstressed snails, the fed snails represent some nutritional stress, and the unfeds greater stress. In similar experiments, which assessed the control of differential catabolism (by measuring oxygen consumption and nitrogenous excretion) during degrowth (Russell-Hunter *et al.*, 1983), highly stressed snails established an effective regime of metabolic compensation (by reducing the proportion of protein catabolism) more rapidly than less stressed snails. Unlike shell mass, tissue biomass is not a static value (see Russell-Hunter and Buckley, 1983, for discussion of this in the actuarial bioenergetics of molluscan productivity). While any individual organism remains alive, its tissue biomass continues to be in turnover. Thus, growth represents a positive value (and degrowth a negative value) for a combined net rate that involves both inputs and outputs as rate functions (Russell-Hunter and Buckley, 1983; Russell-Hunter *et al.*, 1983).

Tissue degrowth in our experiments was paralleled by abnormal patterns of radular secretion. This was manifest in several ways. Smaller lateral, marginal, and rachidian teeth; irregular (malformed) lateral, marginal, and rachidian teeth; and missing marginals were readily apparent (Fig. 1c). Most consistently, tissue degrowth was correlated with an increase in the number of radular rows per unit ribbon length. At 40 days it was apparent that either (1) production of subradular membrane by the membranoblasts and transport by the inferior epithelium had slowed, or (2) the production of radular teeth by the odontoblasts had hastened. Regardless of the relative contributions of these alternative processes, the result is the same. An obvious zone (Figs. 1, 4) of denser row-packing has been created. These observations, after confirmation from field analysis, suggest that activity of membranoblast and odontoblast cell lines is differentially impaired during periods of food deprivation. The nature of the control mechanism regulating these cells is still uncertain so it is not possible to determine how food deprivation influences the results documented here. However, membranoblast activity is reduced to a greater extent than that of the odontoblasts during periods of sustained stress, and this differential secretory response produces the characteristically packed rows. The fact that there are no observable ecophenotypic effects on tooth shape (thought to be under rigid genetic control in

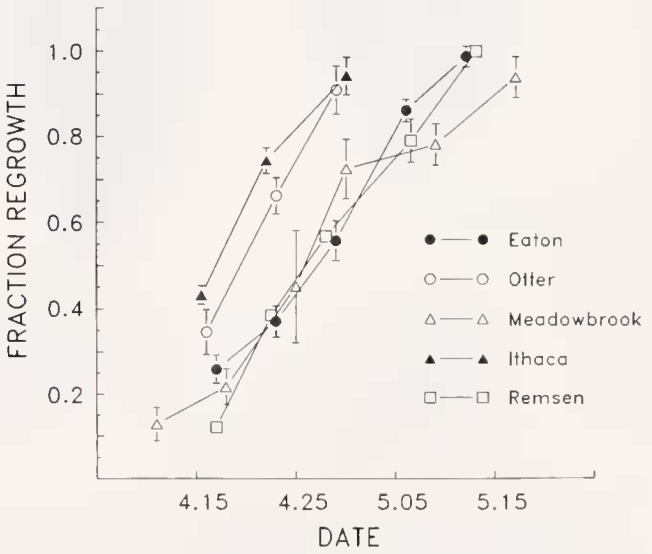

Figure 3. Return to normal radular secretion in spring in five natural populations of *Helisoma*, demonstrating recovery from radular row-packing overwinter (and from presumptive overwinter tissue degrowth). Vertical bars are 95% confidence limits of each mean.

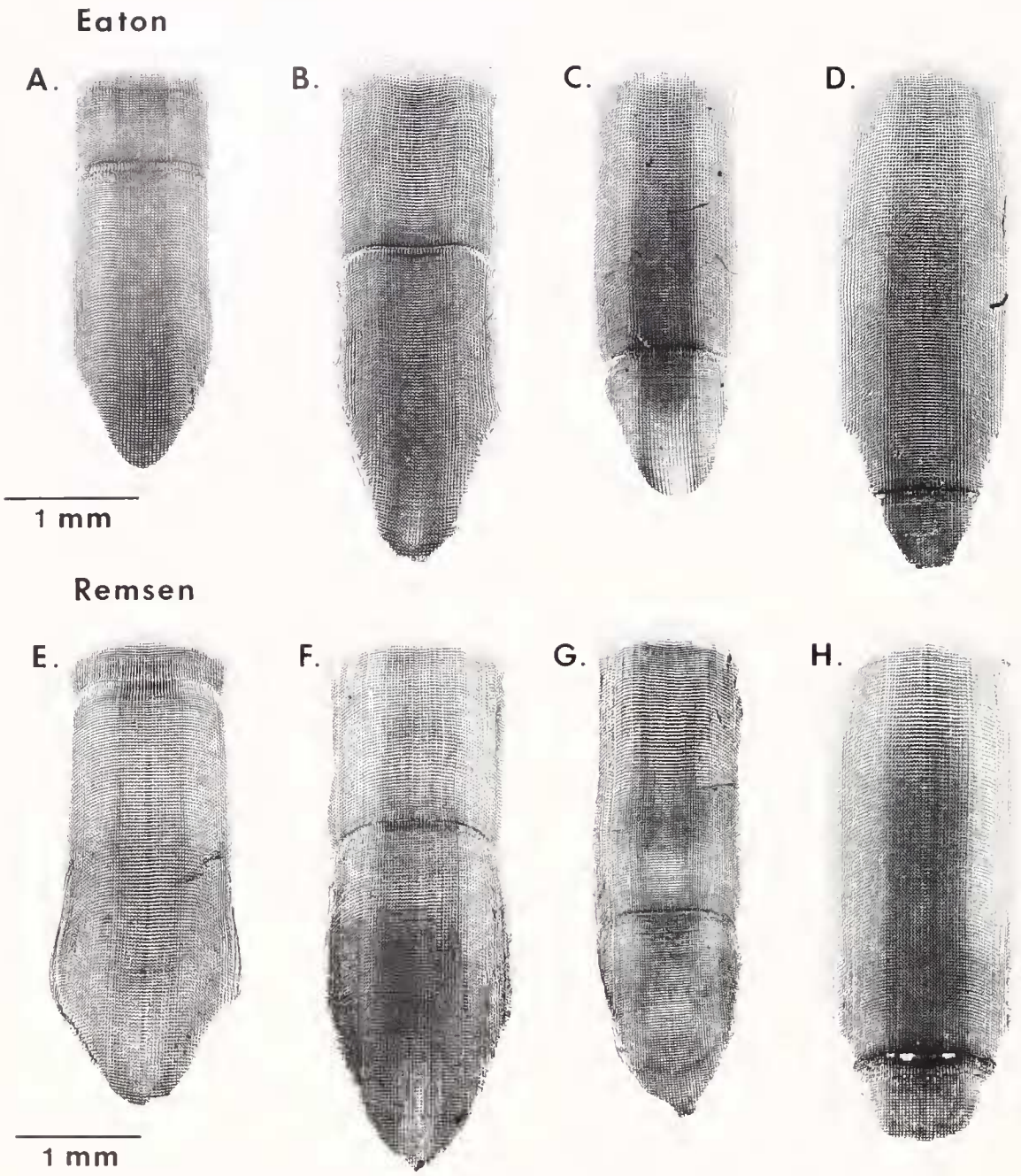

Figure 4. Untouched photographs showing patterns of return to normal radular secretion in two natural stocks of *Helisoma trivolvis*. Note that the most recently secreted radular rows are at the top of each photograph. Radulae from Eaton Reservoir (top, A–D, left to right) represent 29%, 40%, 61%, and 80% regrowth (as fraction new rows of total rows). Radulae from Silver Lake, Remsen (bottom, E–H, left to right) represent 17%, 36%, 53% and 76% regrowth. Samples from both sites were taken at weekly intervals beginning 4.22.85. Actual sizes are: Eaton (top, left to right), 3.1, 3.7, 3.2, and 3.8 mm, Remsen (bottom, left to right), 3.7, 4.3, 3.8, 3.9 mm.

each stock or population; Smith, 1987, 1989) emphasizes the unique significance of row-packing as a predictable response to environmental stress.

In one respect, that of the time sequence of return to normal tissue growth after stress, the radular record of row-packing can be more useful than any assessments based on shell-tissue ratios. In field studies of tissue degrowth (Russell-Hunter *et al.*, 1984), one pond stock of *Helisoma* recovered from 41.3% average degrowth to only 32.8% over three months in spring. In another stock

(from a highly eutrophic lake), overwinter degrowth was eliminated (47.1% net growth) in two spring months. The data on radular recovery (corresponding to regrowth) from the five sites sampled during this study not only show that the process was complete within 25–43 days, but also indicated its temporal sequence in stages.

As noted above, there are two significant aspects to the concurrence of abnormal radular secretion and of tissue degrowth as consequences of starvation stress. The first concerns a matter of fundamental biology in attempting to deduce the control mechanisms involved and, ultimately, the sequence of causality. All patterns of response to environmental stress have evolved to increase the fitness of individuals, and all basically require (i) receptors monitoring changes in the rate of abiotic and physiological parameters, (ii) some system capable of integrating such inputs, and (iii) effector tissues that carry out the response. In the case of the response to starvation in gastropods, we have quantified for (iii) several kinds of effects, we can deduce something of (ii), but we are almost completely ignorant of (i) in specific terms. At the very least we know that the simultaneous effects include both abnormal radular secretion and general tissue degrowth. The former involves both absolute and relative reductions of the secretory activity of membranoblasts. The latter involves not only highly reduced levels of general catabolic activity but also a metabolic shift towards relatively higher turnover of nonprotein carbon. As has been noted (Russell-Hunter, 1985), such controlled differential catabolism can be considered an appropriate parsimony in the net flow through of amino acids (rather than as the defense of a static protein biomass).

There are obvious elements of adaptive conservation in the fact that odontoblast activity is less reduced than membranoblast activity, and in the preservation (relatively) of structural proteins in the tissues. Both differential processes are adaptive in their potential to accelerate return to normal secretion and tissue regrowth when the period of stress has ended. Parenthetically, it should be noted that this capacity for controlled tissue degrowth [increasing individual survivorship under certain environmental conditions by a decrease in individual energy content (Russell-Hunter, 1985; and references therein)] compels reconsideration of certain fitness predictions from simple models of age structure and energy partitioning between growth and reproduction (see for example, Williams, 1966; Tinkle and Hadley, 1975; Browne and Russell-Hunter, 1978).

It seems likely that the ganglia of the snail's central nervous system are involved in integration after the onset of starvation stress. It is unlikely that the integrating system is linked neurally to the rate-controlling cells for membranoblast secretion and those of differential protein catabolism, and barely possible that a specialized endocrine tissue is involved. It can be postulated that the most likely link is through neurosecretory cells. Other systems of integrated control in molluscs involve neurosecretion. For example, sex change in *Crepidula* (Russell-Hunter *et al.*, 1971), and cyclic reproductive behavior in high littoral snails (Price, 1979) involve neurosecretion. Despite the degree of integration of the response to overwinter starvation, it may not be appropriate to term this a diapause, since it is less obligate and more plastic in these snails than in those nematodes and insect larvae from similar habitats for which an innate and essential seasonal diapause has been described. However, there is integration of responses (probably involving neurosecretion), and there can be no question either of abnormal radular secretion (row-packing) *causing* tissue degrowth or even of tissue degrowth *causing* row-packing directly. Although the common cause of both sets of responses appears to be the stress of starvation, these statements belong within David Hume's (1748) regularity theory of causation, which remains appropriate for the logical description of such biological sequences, despite being currently unfashionable among many professional philosophers.

Conclusions from these experimental data have a second significance to applied biology: the possibility of a retrospective detection, in the field, of earlier periods of stress affecting natural populations. Just as the trunks of long-lived forest trees can record in their rings the historical sequence of drought years and of minor forest fires, so the shells of long-lived bivalve molluscs (Clark, 1976; Mallet *et al.*, 1987; Peterson *et al.*, 1985) can record, in their growth rings, a history of severe winters. Radular records of degrowth periods as zones of modified toothrow secretion may provide a history of more recent environmental stress. This may be of applied value in some gastropod stocks by using comparative spring samples of radulae from known populations to assess relative levels of overwinter starvation, and thence to predict productivity for the rest of the year. In addition, similar radular records could be useful in assessing the metabolic stress of a transient period of pollution (such as an oil spill) on populations of freshwater or marine littoral gastropods, even if no records had been obtained *before* the populations were stressed.

Acknowledgments

Work was supported by grants from the Senate Research Committee of Syracuse University (D.A.S. and W.D.R-H.) and the Theodore Roosevelt Memorial Fund (D.A.S.). Preparation of this manuscript was supported by the Treves and Carscallen Funds of Wabash College. This is contribution #102 of the Upstate Freshwater Institute.

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